REMARKS

In response to the Office Action mailed September 10, 2003, Applicants respectfully request reconsideration of the above-identified application in view of the amendments and remarks presented herein.

Applicants acknowledge the Office Action's remarks in "Paragraph 2." As per conversation with the Examiner, all cited references have been considered.

Claims 1, 3 and 13 have been amended. The claims have been amended to correct grammatical and typographical errors. Support for the amended claims can be found throughout the disclosure. Claim 2 has been canceled.

Claims 1, 3 and 5-15 are pending after entry of this amendment.

Claims 1-3 and 5-15 were rejected under 35 USC 112, second paragraph for the reasons set forth in the Office Action. In view of the amendments, and remarks set forth herein, the rejections are traversed.

Specifically, Claim 1 was rejected because, according to the Office Action, the preamble of the claim does not correlate with the body of the claim. Applicants have amended the claim so that the preamble of the claim and the body of the claim recite a method for determining the absolute quantity of a biopolymer. Reconsideration and withdrawal of the rejection are respectfully requested.

Claim 1 was rejected because, according to the Office Action, the phrase "...such as..." renders the claim indefinite. Applicants have deleted the clause. Reconsideration and withdrawal of the rejection are respectfully requested.

Claim 1 was rejected because, according to the Office Action, the phrase "...crude solution..." renders the claim vague and indefinite. Applicants traverse the rejection based upon the arguments presented herein.

The term "...crude..." is well-known to one skilled in the art (*see*, for example A. Datta (1992) J. Biol. Chem. 267:728-736; C.D. Muller and F. Schuber (1986) Anal. Biochem. 152:167-171 and H.A. Barker, E.R. Stadtma, A. Kornberg (1955) Methods Enzymol. Vol.1, pp. 599-602 copies of which are enclosed with this Response). Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

Claim 1 was rejected under 35 USC 112, second paragraph, because, according to the Office Action, parts (c) and (e) of the claim have insufficient antecedent basis. Applicants have amended the claim to overcome the deficiencies. Accordingly, reconsideration and withdrawal of the rejections are herein requested.

Claims 1-3, 5-7 and 11-15 were rejected under 35 USC 102(e) as being anticipated by Aeb rsold et al (US 2002/0076739). These rejections are traversed based upon the amendments to the claims and remarks presented herein.

A *prima facie* case of anticipation requires that each and every limitation is disclosed in a single reference (*see* MPEP 2131 et seq). Claim 1 discloses a method for determining an "...absolute..." quantity of a biopolymer. According to the current invention, an absolute quantity is determined by using, as a reference, a well-characterized and quantitatively defined compound (*see*, for example, disclosure). Aebersold, does not disclose an absolute quantity but, at paragraph 0032, compares one state arbitrarily set as a reference and determines quantity based upon the arbitrary comparison[1]. Accordingly, Aebersold does not disclose or suggest an absolute quantity determination and does not set forth a *prima facie* case of anticipation. Reconsideration and withdrawal of the rejections are respectfully requested.

Claims 2-3 and 4-15 were rejected under 35 USC 102 or 35 USC 103 for reasons found in the Office Action. Each of the above-mentioned claims is dependent upon Claim 1, and patentable, as set forth above. Accordingly, the rejections are deemed moot, except as to claim 2 which has been canceled, and reconsideration and withdrawal of each of the rejections are respectfully requested.

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance and issuance of a formal Notice of Allowance is

[1] In the cited passage, Aebersold, in fact, denotes the quantity as relative, not absolute.

respectfully requested. Examiner Counts is invited to contact Applicants at (650) 846-7544 if there are additional questions/concerns.

Respectfully submitted,

Date: January 10, 2004

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 2, Issue of January 15, pp. 728-736, 1992
Printed in U.S.A.

Purification and Characterization of a Novel Protease from Solid Substrate Cultures of *Phanerochaete chrysosporium*

(Received for publication, August 1, 1991)

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The white-rot basidiomycete *Phanerochaete chrysosporium* is mostly known for its extracellular ligninolytic enzymes. In this paper, the purification and characterization of a novel extracellular protease secreted by this fungus in solid substrate cultures under ligninolytic conditions are described. The purification steps included extraction of enzymes from the wood substrate, concanavalin A-Sepharose chromatography, anion-exchange chromatography (Mono Q), and size-exclusion chromatography (Superose 12). The purified protein migrates with M_r = 40,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It has an isoelectric point of 5.6 and a sharp pH optimum at 4.0. The protease is totally inhibited by Hg^{2+}, *p*-hydroxymercuribenzoic acid, and *N*-bromosuccinimide, but is insensitive to phenylmethanesulfonyl fluoride, pepstatin A, and EDTA. Its amino acid composition and NH_2-terminal sequence have also been determined. The sequence data and the binding of the protease to concanavalin A indicate that the protease is a glycoprotein. The protease differs in its physicochemical properties and its response to inhibitors from other extracellular proteases previously found in another strain of *P. chrysosporium*. The data suggest that it has properties of both aspartate-type and thiol-type proteases.

The white-rot basidiomycete *Phanerochaete chrysosporium* has received extensive attention because of its powerful lignin-degrading activity. This ligninolytic property of this microorganism also makes it an attractive candidate for industrial applications (1) and for the treatment of hazardous organic compounds (2). The biochemistry of lignin degradation by *P. chrysosporium* is a complex process involving a series of enzymatic and non-enzymatic reactions. Evidence indicates that major extracellular enzymes that are involved in this process are lignin peroxidase, manganese-dependent peroxidase, and hydrogen peroxide-producing enzymes, including glucose oxidase (intracellular) and glyoxal oxidase. The *in vitro* reactions catalyzed by these enzymes have been worked out in detail (for reviews, see Refs. 3–6), although their *in vivo* coordination is not clearly understood. There is some evidence that certain ligninolytic enzymes may act synergistically (7).

In a recent study of the ligninolytic system of *P. chrysosporium* in solid substrate cultures (8), I found that this fungus also expresses a substantial amount of proteolytic activity. Very little is known about the extracellular proteases secreted by *P. chrysosporium*, despite the fact that production of extracellular proteolytic enzymes is common among fungi (9). Eriksson and Pettersson (10) reported the purification and partial characterization of two proteases from *Sporotrichum pulverulentum* (=*P. chrysosporium*) grown with cellulose as a carbon source. These two acidic proteases were found to activate the endo-1,4-β-glucanases present in the cultures. Very recently Grethlein and co-workers (11, 12) studied the effect of culture conditions on protease activity in ligninolytic cultures of *P. chrysosporium*.

This paper reports the purification and characterization of a protease discovered in wood substrate cultures of *P. chrysosporium*. Solid substrate culture conditions are close approximations to the *in vivo* growth conditions of these microorganisms, since wood is their natural habitat. It is also noteworthy that solid substrate culture conditions can switch on enzyme systems that differ from their liquid culture counterparts (8). The physico-chemical properties of the protease described here differ significantly from those reported by Eriksson and Pettersson (10). The enzyme, which has been purified to homogeneity, is an acidic protease with an unusual response profile to the inhibitors studied. Its amino acid composition and NH_2-terminal sequence were also determined.

MATERIALS AND METHODS

Culture Conditions and Extractions of Enzymes—*P. chrysosporium* BKM-F-1767 (ATCC 24725) was grown in coarse refiner mechanical pulp from aspen (*Populus tremuloides*) in polypropylene containers (8).

At the end of the 3-day incubation period, the pulp, soaked in 10 mM sodium acetate buffer (pH 4.5), was pressed with a hydraulic press to extrude most of the fluid out of the solid matrix. The exudate was subsequently filtered, concentrated, and frozen in small portions for further purification and characterization (8).

Enzyme Assays—Protease activity was measured with Azocoll (Sigma) as the substrate (11). The protease was incubated with 20 mg of substrate in 50 mM sodium acetate (pH 4.5), with periodic shaking in a tube at 37 °C for 30 min, unless otherwise stated. The total volume of the reaction mixture was 1 ml. At the end of the incubation period, the reaction was stopped by adding 10% trichloroacetic acid, the tube was centrifuged at 12,000 × *g* for about 8 min, and the absorbance of the supernatant solution was read at 520 nm. The blank contained the substrate and buffer, and its reading was subtracted from the sample reading. One unit of protease activity was defined as the amount of enzyme that catalyzes the release of enough azo dye to give a change in absorbance at 520 nm of 0.001 in 30 min. General peroxidase activity was determined with *o*-tolidine (3,3'-dimethylbenzidine) as the substrate (14).

* This work was supported by Biopulping Consortium, composed of 20 pulp and paper and associated companies, the University of Wisconsin Biotechnology Center, and the Forest Products Laboratory, United States Department of Agriculture. I acknowledge the University of Wisconsin Biotechnology Center for protein sequencing work and the Protein/Nucleic Acid Shared Facility of the Medical College of Wisconsin, supported by National Institutes of Health shared instrumentation Grant NIH-RR-03326, for amino acid analyses. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Enzyme Purification—The purification steps, except the final chromatography, were identical to those reported in detail by Datta *et al.* (8). In a typical purification procedure, an aliquot of the crude concentrate, approximately 5 ml, was adjusted with stock NaCl solution to a final concentration of 0.5 M NaCl. The extract was passed through an affinity column of concanavalin A-Sepharose (Sigma) and washed with 10 mM sodium acetate (pH 6.0) containing 0.5 M NaCl. The bound proteins were then eluted with the above buffer containing 0.5 M α-methyl-D-mannopyranoside (Sigma). Fractions with protease activity were pooled.

The pool was concentrated, desalted through a Sephadex G-25 column (0.5 × 7.0 cm; Bio-Rad), and chromatographed on a Mono Q anion-exchange column (Pharmacia LKB Biotechnology Inc.) interfaced with a fast protein liquid chromatography system (Pharmacia). A gradient from 10 mM to 1 M sodium acetate (pH 6.0) was applied over a 40-min period at a flow rate of 1 ml/min. The eluate was monitored at 405 and 280 nm with a UV-VIS monitor (Pharmacia). The protease peak, which absorbed at 280 nm only, was eluted later than the peroxidase peaks, which absorbed both at 280 and 405 nm. The peaks were well separated, and assays of the collected fractions (1 ml) showed no overlap of protease and peroxidase activities.

The protease pool from the Mono Q column was concentrated at 4 °C approximately 10-fold using an Amicon stirred cell and Amicon Diaflo Ultrafiltration membrane (Amicon, Danvers, MA) with a M_r 10,000 cutoff. The concentrated pool was finally subjected to size-exclusion chromatography on a Superose 12 column (Pharmacia) connected to the fast protein liquid chromatography system. Protease samples (0.2 ml) were injected and eluted with 50 mM sodium acetate (pH 4.5) at a flow rate of 0.3 ml/min. The fraction size was 0.3 ml. The volume of pooled fractions was approximately 1.6 ml.

SDS[1]-Gel Electrophoresis—Chromatographic fractions were analyzed by SDS-polyacrylamide slab gel electrophoresis (PAGE) and were stained with Coomassie Blue (8). Pharmacia PhastSystem gel electrophoresis was also used with precast SDS-polyacrylamide gels (homogeneous, 12.5%; Pharmacia), which were subsequently stained with Coomassie Blue.

Isoelectric Focusing—Proteins were analytically focused on Serva precasts (pH range 3–6, Serva, Heidelberg, Federal Republic of Germany), using a Bromma 2117 Multiphor apparatus (LKB, Sweden). Standards (Sigma) had pI values of 3.55 (amyloglucosidase), 4.55 (soybean trypsin inhibitor), 5.13 (β-lactoglobulin A), and 5.85 (carbonic anhydrase B). The proteins were stained with Coomassie Blue.

Protein Electroblotting, Sequencing, and Amino Acid Composition—Fractions pooled from the Mono Q column (on the basis of absorption profiles, SDS-PAGE results, and enzymatic assays) were concentrated to approximately 80 μg/100 μl by using Centricone spin concentrators (Amicon). The concentrated sample was electrophoresed on SDS-polyacrylamide gels and electroblotted onto a nitrocellulose or polyvinylidene difluoride (Millipore) membrane at 15 °C by the method of semidry electrophoretic transfer with the Pharmacia PhastSystem. A typical transfer was carried out at 20 V, with 25 mA of current per gel for approximately 30 min. The transfer solution contained 25 mM Tris, 192 mM glycine, and 20% methanol.

The protease was prepared for sequencing by electroblotting to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) (15). Protein sequencing was performed at the University of Wisconsin Biotechnology Center with an Applied Biosystems (ABI) gas-phase 475A Protein Sequencer (16). Sequencing cycles designed for polyvinylidene difluoride (17) were used with on-line phenylthiohydantoin-derivative separation on an ABI 120A analyzer. Amino acid analyses were performed with a Beckman 6300 amino acid analyzer at the Protein/Nucleic Acid Shared Facility, Medical College of Wisconsin, Milwaukee.

pH Profile Studies—The protease sample was incubated with Azocoll at different pH values at 37 °C for 2 h and 15 min and then assayed as described above. The buffers used for different pH ranges were the following: for pH range 3–5, 50 mM sodium acetate; for pH range 6–8, 50 mM potassium phosphate; and for pH 9, 50 mM sodium borate.

Inhibition Studies—All inhibitors were diluted from 5 mM stock solutions in 50 mM sodium acetate (pH 4.5) to a final concentration of 1 mM, except *p*-hydroxymercuribenzoic acid (sodium salt) (HMB), which was first dissolved in 0.1 N NaOH and then further diluted into the acetate buffer. The concentration of the protease was adjusted such that its activity corresponded to an absorbance change of 0.2 per 30 min at 520 nm in the regular assay, as described above. The protease (approximately 0.2 μg) was incubated with the inhibitors for 30 min at 37 °C and then assayed with Azocoll after 1.75 h of incubation with the substrate at 37 °C.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide slab gel electrophoresis; HMB, *p*-hydroxymercuribenzoic acid (sodium salt); PMSF, phenylmethanesulfonyl fluoride.

RESULTS

Extraction and Purification

Extraction—The inoculated aspen pulp was harvested on day 3 when the level of colored compounds in the extract was low. The levels of extracellular lignin-degrading peroxidases of this organism were also found to be high on day 3. The enzymes from inoculated pulp were pressed out with a hydraulic press, which generated enough pressure to recover most of the enzymes. I estimated approximately 4 mg of total protein in the crude extract/1,000 g dry weight of pulp. The uninoculated wood extract did not show any proteolytic activity. The use of a hollow-fiber filtration system removed most of the high M_r interfering compounds (8). The extract was subsequently concentrated as much as 190-fold with a tangential flow ultrafiltration system with a cutoff M_r of 10,000.

Concanavalin A-Sepharose Chromatography—The protease, along with the peroxidases from the crude extract, was isolated by using an affinity column of concanavalin A bound to Sepharose. The protease and other glycoproteins (manganese-dependent peroxidase, lignin peroxidase, and glyoxal oxidase) were mostly recovered on elution with a solution of 0.5 M α-methyl-D-mannopyranoside. No enzymatic activity was detected in the flow-through, although a substantial amount of residual colored material was removed in the process. Most of the protease and peroxidase activities were recovered in the same fractions. These fractions were pooled, concentrated, and desalted through a Sephadex G-25 column prior to loading onto an ion-exchange column.

Ion-exchange Chromatography—To fractionate the proteins further, an anion-exchange column (Mono Q) connected to a fast protein liquid chromatography system was used. The peroxidases and protease were well resolved with a gradient of sodium acetate (pH 6.0). A typical chromatogram is shown in Fig. 1. Assays and SDS-PAGE of the fractions showed that the peroxidases were eluted first, followed by the protease.

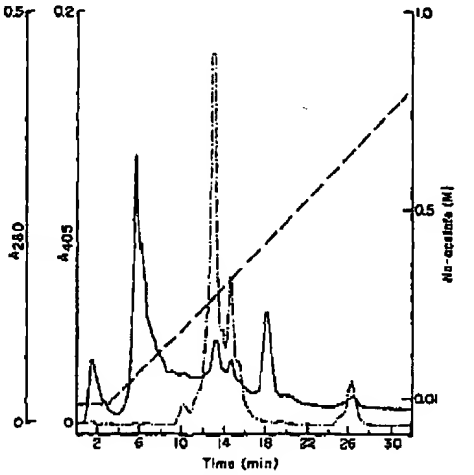

FIG. 1. **Mono Q chromatogram of the crude extract.** Absorbances at 280 and 405 nm are shown by the *solid line* and the *dotted line*, respectively. For details of the chromatography, see "Materials and Methods."

The protease was eluted at a retention time of 18 min. The peroxidases, being heme proteins, absorbed both at 280 and 405 nm, whereas the protease absorbed only at 280 nm. Fractions with protease activities were pooled and concentrated with Amicon spin column concentrators.

Size Exclusion Chromatography—The concentrated pool of Mono Q fractions containing the protease was injected into a Superose 12 gel filtration column and eluted with 50 mM sodium acetate (pH 6.0). The protease was eluted under a well resolved single peak. The purification steps are shown in Table I. The SDS-PAGE of purified protease showed a single band (Fig. 2).

Determination of Molecular Weight

The molecular weight of the protease was determined from its migration distance on SDS-polyacrylamide gel. Estimation was based upon the calibration curve constructed with known molecular weight markers. The molecular weight was found to be approximately 40,000.

pH Profile and Isoelectric Point

The pH optimum of the protease was determined from activity measurements over a broad pH range. The enzyme was found to be most active in the acidic pH range, with maximum activity at pH 4.0 (Fig. 3). The activity was found to decline sharply on either side of the peak. The isoelectric point of the protease was determined to be 5.6.

Reactions with Inhibitors

The protease was tested against a wide array of inhibitors. Its residual activity was measured after incubation with each inhibitor for a fixed length of time, and this activity was compared with the control which was incubated for the same length of time at the same concentration without any inhibitor. The results (shown in Table II) revealed some interesting features: the protease was found to be mostly insensitive to commonly used protease inhibitors, including phenylmethanesulfonyl fluoride (PMSF), pepstatin A, EDTA, and azide. Cyanide was found to inhibit it marginally. Among the heavy metals studied, only Hg^{2+} was found to be effective in inhibiting the enzyme activity. Cu^{2+} and Ag^{2+} were noninhibitory. *N*-Bromosuccinimide and HMB inactivated the enzyme completely, although some residual activity was seen when dithiothreitol was included during incubation with the latter compound.

Amino Acid Composition and NH_2-terminal Sequence

The amino acid composition of the protease (Table III) shows that the enzyme has very low levels of methionine and histidine. The NH_2-terminal sequence, determined with confidence up to 28 residues, shows a potential *N*-glycosylation site (Table IV).

Interaction with Extracellular Enzymes

When a crude protein extract from *P. chrysosporium* cultures was incubated with the protease for 1 h at 37 °C, no enhancement in the activity of lignin peroxidase, manganese-dependent peroxidase, or glyoxal oxidase was observed under the given experimental conditions. No clear evidence of lignin peroxidase (isolated from liquid cultures) (18) degradation was seen under identical conditions. The lignin peroxidase activity remained unaltered by protease treatment, and no fragmentation of lignin peroxidase was observed upon SDS-PAGE.

DISCUSSION

Proteolytic enzymes play major roles in fungal physiology, including functions such as germination and sporulation. Under ligninolytic conditions, they can scavenge nitrogen from woody cell walls and amino acids from denatured fungal proteins in a nitrogen-deficient environment. They can also activate zymogenic enzymes by limited proteolysis and possibly can release from fungal cell walls enzymes that attack wood polymers (10). Recent observations by Dosoretz *et al.* (11) suggest the involvement of proteases in the decline of lignin peroxidase activity during secondary metabolism in liquid cultures of *P. chrysosporium*. These investigators found an inverse correlation between overall lignin peroxidase activity and protease activity in liquid cultures and observed that glucose was a key regulatory nutrient in the control of protease activity.

My observations show that a significant level of protease activity occurs in solid substrate cultures of *P. chrysosporium*. Although the total amount of protein present in the extract was low, at least 300 units of activity were detected per 1,000 g (dry weight) of inoculated aspen pulp. Initial extraction of the enzymes from solid substrate cultures was complicated by the presence of high amounts of colored polyphenolics and viscous wood hemicelluloses. However, the use of younger cultures and subsequent filtration with high M_r cutoff membranes greatly reduced the amount of these interfering compounds. As a result, the successive chromatographic steps were easier to carry out and yielded a pure protease, with maximum purification occurring during the ion-exchange step. The apparent increase in activity yield after the Superose 12 chromatography probably reflects the removal of some of the naturally occurring inhibitors from the crude extract. The method of squeezing the extract out of the pretreated pulp was shown to be adequate by spectroscopic and other techniques (data not presented). Manganese-dependent peroxidase and glyoxal oxidase were two other extracellular enzymes that were found to be relatively abundant under the experimental conditions.

Since the protease releases azo dye from Azocoll, it is most likely an endoprotease. Like most other fungal proteases, it is acidic, with a very sharp optimum at pH 4.0. Its molecular weight falls within the range of most acid proteases (30,000–

TABLE I

Purification scheme of the protease

Step	Volume	Total protein	Total protease activity	Specific activity	Purification factor	Yield
	ml	A_{280} *units*	A_{280} *units*	A_{405}/A_{280}		%
Crude concentration	2	22.4	131.6	5.8	1	100
Concanavalin A-Sepharose (after concentration)	0.27	1.1	44.9	40.82	6.9	34.1
Sephadex G-25	0.70	0.8	32.1	40.1	6.8	24.4
Mono Q	1.9	0.02	8.4	420	71.4	6.4
Superose 12	1.6	0.02	17.9	895	152.2	13.6

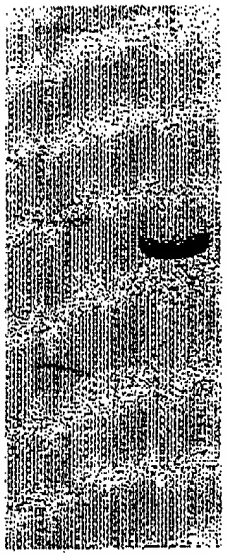

FIG. 2. SDS-PAGE of purified protease. Approximately 0.5 μg of protein was loaded on a 12.5% homogeneous SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. The molecular weight markers are (from top to bottom): 97,400, 66,200, 42,700, 31,000, and 14,400.

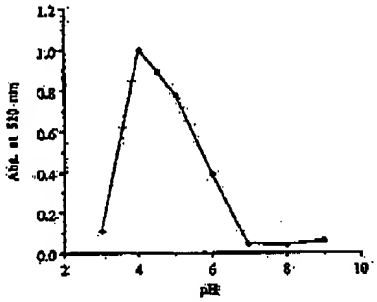

FIG. 3. The pH profile of the protease. See "Materials and Methods" for details.

TABLE II

Effect of inhibitors on protease activity

Inhibitor	Activity remaining %
Control	100
Azide	95
NaCN	68
$CuSO_4$	100
$AgNO_3$	100
$HgCl_2$	0
p-Hydroxymercuribenzoate	0
p-Hydroxymercuribenzoate (+2 mM dithiothreitol)	18
EDTA	100
PMSF	88
Pepstatin A	93
N-bromosuccinimide	0

45,000) (19). The *P. chrysosporium* protease has a slightly higher pI (5.6) than most other acidic proteases (typical pI values: 3–5); however, Tapia *et al.* (20) have reported an extracellular acid protease from *Sporotrichum dimorphosporum* with pI = 7.4.

This work has revealed interesting information about the

TABLE III

Amino acid composition of protease

Residue	Number	Mol %
Cysteine	3	0.66
Asparagine/aspartic acid	48	11.93
Threonine	32	8.11
Serine	45	11.62
Glutamine/glutamic acid	30	7.51
Proline	23	5.69
Glycine	46	11.49
Alanine	49	12.20
Valine	24	6.09
Methionine	1	0.19
Isoleucine	18	4.40
Leucine	30	7.49
Tyrosine	9	2.14
Phenylalanine	28	7.09
Histidine	1	0.20
Lysine	8	1.92
Arginine	5	1.27

TABLE IV

NH_2-terminal sequence of the protease

The sequence was determined up to 28 residues with confidence. The underlined residues indicate a possible *N*-glycosylation site. Two residues remain unidentified (X).

10 20 28

A-S-E-S-T-S-A-V-T-P-A-X-X-E-S-L-Y-G-Y-P-T-T-R-A-T-Q-S-S

sensitivity of the *P. chrysosporium* protease toward different inhibitors. It was possible to remove the protease activity almost completely by batch treatment of the crude concentrate with α_2-macroglobulin. However, the enzyme was found to be insensitive to the usual protease inhibitors, such as PMSF, EDTA, and pepstatin A. Dosoretz *et al.* (11) observed a decline in overall protease activity when PMSF was added to growing fungal cultures. Although the majority of fungal acid proteases are pepstatin A-sensitive, a few have been reported to be insensitive to pepstatin A, for example the proteases from *Aspergillus niger* (21), *Lentinus edodes* (22), *S. dimorphosporum* (20), and *Trichoderma reesei* (23). Many of these proteases are, however, sensitive to *N*-bromosuccinimide, and my observations with this inhibitor show that the *Phanerochaete* enzyme also displays this characteristic. These findings suggest that the *P. chrysosporium* protease belongs in the subclass of aspartate proteases, which have aspartic acids as the active site residues, as in pepsin (24). On the other hand, its sensitivity toward HMB is more consistent with the behavior of a thiol protease. As a matter of fact, some acid proteases have been shown to be of the thiol type (19). In this respect, the protease described in this report behaves like those from *Sporotrichum pulverulentum* (10). The acidic proteases from *S. pulverulentum* were also found to be sensitive to HMB, but in contrast with my observations, the activity of this enzyme was almost completely restored in presence of dithiothreitol. As regards all other physico-chemical properties, including molecular weight, isoelectric point, pH optimum, and response to other inhibitors, the protease from solid substrate cultures of *P. chrysosporium* was found to be entirely different from the proteases characterized from *S. pulverulentum*. I did not observe zymogenic activation of any of the known ligninolytic enzymes of *P. chrysosporium* by this protease under the given conditions. However, Eriksson and Pettersson (10) have reported an increase in endoglucanase activity upon treatment with the protease that they isolated from *S. pulverulentum*.

Amino acid analyses and NH_2-terminal sequence analyses for other proteases from ligninolytic fungi have not been

reported and are pertinent. Here, separate estimates of glutamine and asparagine were not possible because of spontaneous deamination of these 2 residues during sample preparation. In addition, the instrument was not equipped for tryptophan analysis. The NH_2-terminal sequence shows clear evidence of a glycosylation site conforming to the general rule Asn-*X*-Thr/Ser (25), which is consistent with the fact that many of the fungal aspartate proteases, including those from *Mucor miehei* (26), *Scytalidium lignicolum* (27), and *Aspergillus oryzae* (28) contain carbohydrates. No significant homology was seen when the sequence was compared with other known sequences using NBRF-Protein and Swiss-Protein databases. This is not surprising, considering the fact that the active site is usually located far from the NH_2-terminal region, and so the NH_2-terminal region does not have to be highly conserved.

At this point, the role of this protease is unclear. However, it is possible that the enzyme could be involved in the intracellular cleavage of the putative lignin peroxidase propeptide (29). Subsequently the protease could be released into the extracellular matrix following fungal autolysis. Very recently, the data by Messner and co-workers (30) suggest a correlation between autolysis and release of extracellular enzymes from fungal hyphae.

It will be interesting to compare further this protease isolated from solid substrate cultures with that from submerged cultures of *P. chrysosporium*. Dosoretz *et al.* (11, 12) studied the effect of environmental conditions on protease activity from liquid cultures of *P. chrysosporium* and the protease-mediated degradation of lignin peroxidase, but no information is available on the physico-chemical properties of their protease. It is possible that these properties might differ depending on the culture conditions.

In summary, I have reported here the purification and characterization of a novel protease from solid substrate cultures of *P. chrysosporium*. It is an extracellular, acidic protease, most likely of the aspartate type. The NH_2-terminal sequence data suggest that the enzyme is a glycoprotein. Its sensitivity toward various inhibitors is unique in some respects. Further work is needed to study the effect of culture conditions on its formation and to understand the nature of its substrate, including specificity and kinetics.

Acknowledgments—I thank T. Kent Kirk of Forest Products Laboratory for many stimulating discussions and critical review of the manuscript and Alan Bettermann for his help in protein electroblotting.

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triethanolamine buffers (0.1 *M*, pH 8.0). At the same concentrations and pH, potassium diethylbarbiturate and citrate buffers cause 50% inhibition, and inhibition by potassium pyrophosphate is about 90%. Relatively high concentrations (0.01 *M*) of ATP and ADP inhibit the enzyme partially.

The enzyme activity is lost upon dialysis and cannot be restored by the addition of known cofactors or crude boiled extracts.

The rate of the arsenolysis reaction is directly proportional to the concentration of CoA over a very wide range (0 to 200 units/ml.). The concentration of CoA needed to saturate the enzyme has not been determined.

The rate of arsenolysis is a linear function of the arsenate concentration over the range of 0 to 0.1 *M*; further, nonlinear increases in rate are observed up to arsenate concentrations of 0.2 *M*.

Effect of pH. The enzyme is active over the pH range of 6.6 to 9.0 with a broad optimum range of 7.4 to 8.4.

Spectrophotometric Measurement of Enzyme. Reaction 1 may be followed directly by measuring the changes in optical density at 232 to 240 mμ that are associated with the formation or cleavage of the thiol ester bond of Ac~SCoA.[9] Since this method requires considerably more CoA (0.1 micromole), it is not recommended as a routine assay procedure.

Equilibrium Constant. The equilibrium constant[10] for reaction 1 is:

$$K = \frac{(\text{Ac}\sim\text{SCoA})(\text{HPO}_4^{--})}{(\text{Ac}\sim\text{P})(\text{CoA})} = 74$$

[9] E. R. Stadtman, *J. Biol. Chem.* **203**, 501 (1953).

[10] An equilibrium constant of 60 ± 20 was reported earlier [E. R. Stadtman, *J. Biol. Chem.* **196**, 535 (1952)], but more recent studies have shown it to be about 74 (E. R. Stadtman, unpublished results).

[99] Coenzyme A Transphorase from *Clostridium kluyveri*

Butyryl~SCoA + Ac ⇌ Butyrate + Ac~SCoA

By H. A. Barker, E. R. Stadtman, and Arthur Kornberg

Assay Method

Principle. The method is based on the decrease in optical density in the 232- to 240-mμ region when the above reaction is coupled with the arsenolysis of Ac~SCoA under the influence of phosphotransacetylase.[1] The net reaction is an apparent hydrolysis of butyryl~SCoA.

[1] E. R. Stadtman, *J. Biol. Chem.* **203**, 501 (1953).

$$\text{Butyryl}\sim\text{SCoA} + H_2O \rightarrow \text{Butyrate} + \text{CoASH}$$

At 232 mμ, the molar extinction coefficient of CoASH is about 55% of that of acyl~SCoA. The difference in the molar extinction coefficients of butyryl~SCoA and its hydrolysis products (ΔE_{232}) is 4.5×10^3 cm.²/mole.

Reagents

Butyryl~SCoA (0.001 *M*). When the solution is adjusted to pH 6 with KOH, the compound is stable for months at −15°.

0.15 *M* KAc–0.33 *M* $KAsO_4$, pH 7.0.

Phosphotransacetylase.[2] Dilute the stock solution to 40 units/ml. with 0.05 *M* Tris-HCl buffer, pH 8.0. The enzyme solution is stable for months at −15°.

Enzyme. Dilute the stock solution to a concentration of 0.05 to 0.15 unit/ml. (See definition below.)

Procedure. Add 0.2 ml. of KAc–$KAsO_4$ solution and 0.1 ml. each of butyryl~SCoA and phosphotransacetylase solutions to 1.0 ml. of water in a quartz cell having a 1-cm. light path and a 1.5-ml. capacity. Then add 0.1 ml. of enzyme solution, and take readings at 232 mμ at 1-minute intervals. The reference cell contains all reagents except butyryl~SCoA and phosphotransacetylase in order to compensate for the high ultraviolet absorption by the enzyme. Since the rate of the reaction declines with time, the enzyme activity is calculated from the optical density change between the first and fourth minutes.

Definition of Unit and Specific Activity. One unit of enzyme is defined as that amount which causes the decomposition of 1 micromole of butyryl~SCoA per 3 minutes. This corresponds to a rate of optical density change (ΔOD_{232}) of 1.0 per 3 minutes at 25° under the above conditions. Specific activity is expressed as units per milligram of protein, determined by the method of Lowry *et al.*[3]

Purification Procedure

Only small purification of CoA transphorase has been achieved by the methods applied until now.[4] However, the best preparations have a reduced nucleic acid content ($OD_{230m\mu}/OD_{260m\mu} = 1.23$) compared to the

[2] E. R. Stadtman, *J. Biol. Chem.* **196**, 527 (1952). The fraction obtained by alcohol precipitation (50 to 58%) is satisfactory; see Vol. I [98].

[3] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951); see Vol. III [73].

[4] A. Kornberg, H. A. Barker, and E. R. Stadtman, unpublished results.

starting material (0.62) and are relatively free of light-absorbing components, in both the visible and ultraviolet regions.

Step 1. Preparation of Crude Extract. 10 g. of dried and ground cells of *Cl. kluyveri*[5] is homogenized with 100 ml. of 0.002 *M* KPO_4, pH 7.4, and incubated with occasional stirring at 30° for 4 hours. The solution is centrifuged at 10,000 r.p.m. for 10 minutes, the supernatant liquid decanted, and the precipitate washed twice with 40 ml. of buffer. The combined supernatant liquid (volume, 140 ml.) retains its activity for months when stored.

Step 2. Protamine Treatment. To 1 vol. of extract from step 1 is added 0.2 vol. of a 1% solution of protamine sulfate (salmine). After 5 minutes at 0°, the solution is centrifuged in the cold, and the precipitate is discarded. To the supernatant solution add 0.2 vol. of 1 *M* $KAsO_4$, pH 7.0, and 0.05 vol. of 1 *M* KAc, and dilute to twice the original volume.

Step 3. Fractionation with Ammonium Sulfate. To the cold enzyme solution of step 2 is added sufficient powdered ammonium sulfate to give 0.45 saturation. After stirring for 5 minutes at 0°, the precipitate is removed by centrifuging at 12,000 r.p.m. The supernatant liquid is adjusted to 0.65 saturation by addition of solid ammonium sulfate, and, after centrifugation, the 0.45 to 0.65 fraction is dissolved in one-fifth the original volume of 0.05 *M* $KAsO_4$, pH 7.0. This preparation loses approximately 25% of its activity in two weeks at −15°.

Step 4. Fractionation with Ethanol. KCl, KAc, and acetic acid are added to the solution derived from step 3 in such amounts as to give final concentrations of 0.2 *M*, 0.02 *M*, and pH 6.3, respectively, when the protein concentration is adjusted to 3.0 mg./ml. Absolute ethanol cooled to −10° is slowly added to the solution, which is stirred continuously, until the alcohol concentration is 31% (v/v). After 5 minutes the precip-

SUMMARY OF PURIFICATION PROCEDURE[a]

Fraction	Total volume, ml.	Units/ml.	Total units	Protein, mg./ml.	Specific activity, units/mg.	Recovery of activity, %
1. Crude extract	80	1.00	80	13.9	0.072	(100)
2. Protamine supernate	90	0.65	58	6.4	0.101	73
3. $(NH_4)_2SO_4$ fraction, 0.45–0.65 satn.	20	2.08	42	11.0	0.190	52
4. Ethanol fraction, 31–49%	20	1.26	25	4.0	0.310	31

[a] H. A. Barker, A. Kornberg, and E. R. Stadtman, unpublished experiments.

[5] E. R. Stadtman and H. A. Barker, *J. Biol. Chem.* **180**, 1085 (1949); see Vol. I [84].

itate is removed by centrifugation, and more alcohol is added to the supernatant solution to give a final concentration of 49%. The solution is centrifuged, and the precipitate is immediately dissolved in the original volume of 0.05 *M* $KAsO_4$, pH 7. All operations are carried out at 0 to −6°. The product of step 4 retains 90% of its initial activity during storage for two weeks at −10°. For some purposes orthophosphate or triethanolamine is preferable to arsenate as a buffer for the final enzyme preparation. See the accompanying table for a summary of the purification procedure.

Properties

Specificity. Crude extracts of *Cl. kluyveri* catalyze the transfer of the CoA moiety of acetyl CoA to formate, acetate, propionate, *n*-butyrate, *n*-valerate, *n*-caproate, *n*-caprylate, vinyl acetate, lactate, and possibly glycolate.[1,6] The reactions are generally reversible. The specificity of the purified enzyme has not been investigated.

Effect of pH. The enzyme shows a pH optimum for activity of pH 6.8 to 7.0. The pH range for activity is from approximately 5.6 to above 8.0.

Effect of Substrate Concentration. Incomplete data indicate that the Michaelis constants for butyryl CoA and acetate are approximately 5×10^{-6} *M* and 7×10^{-3} *M*, respectively.

Heat Stability. When crude extracts are heated for 5 minutes at 55, 60, and 65°, the enzyme loses approximately 40, 52, and 78%, respectively, of its activity. Since phosphotransacetylase is much more thermolabile, preparations heated for 15 minutes or longer at 60° have a low content of this contaminating enzyme.[1]

[6] I. Lieberman and H. A. Barker, unpublished results.

[100] Deacylases (Thiol Esterase)

Succinyl CoA Deacylase

$$\text{Succinyl}\sim\text{SCoA} + H_2O \rightarrow \text{Succinate} + \text{HS-CoA}$$

By JOHN GERGELY[1]

Assay Method

Principle. The method described below[2] is based on the following facts. The enzymatic reduction of DPN by α-ketoglutarate (KG),[3] ac-

[1] Established Investigator of the American Heart Association.
[2] J. Gergely, P. Hele, and C. V. Ramakrishnan, *J. Biol. Chem.* 198, 323 (1952).
[3] KG will be used throughout as an abbreviation of α-ketoglutarate or the free acid.

ANALYTICAL BIOCHEMISTRY 152, 167–171 (1986)

Fluorometric Determination of Polystyrene Latex: Application to the Measurement of Phagosomes and Phagocytosis

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Received July 3, 1985

Intrinsic fluorescence of polystyrene dissolved in organic solvents such as 1,2-dimethoxyethane was used to develop a sensitive method for the quantification of polystyrene latex beads. This method allows the assay of latex in the microgram range and is one order of magnitude more sensitive than the conventional spectrophotometric method. The fluorometric technique was used in the quantification of phagocytic latex particle uptake by macrophages and in the quantification of isolated phagosomal fractions.

KEY WORDS: fluorescence; latex beads; phagocytosis; macrophages.

Phagocytosis is an important phenomenon which serves microorganisms for nutritional needs or allows specialized cells such as macrophages to eliminate unwanted materials [e.g., bacteria, aged cells (1–3)]. It involves an invagination of a portion of the cell surface and the formation of phagosomes, i.e., cytoplasmic vesicles, where the ingested particle is closely surrounded by the plasmalemma-derived membrane. *In vitro* study of phagocytosis generally requires quantification methods. Numerous assay systems have been developed (4) and among them phagocytosis of latex beads has been widely used. The latex beads are easy to visualize under the light microscope and their density allows facile access to phagosomes by floatation in density gradients (5,6). The quantification of the beads was generally determined spectrophotometrically (1) after their extraction by organic solvents according to the method originally described by Roberts and Quastel (7). The sensitivity of this method can be limiting. We have found that polystyrene latex beads in phagosomes can be easily quantified by a fluorometric method which allows the assay of latex in the microgram range.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials. Monodisperse preparations of polystyrene latex beads of 1.1-μm diameter were obtained from Sigma Chemical Company or from Serva. Hanks' balanced salt solution (Ca^{2+}- and Mg^{2+}-free), Medium 199, Dulbecco's modified Eagle's medium (DMEM)[2], and calf serum were purchased from Gibco. Heparin and tuftsin were from Sigma. The solvents used in the study were of the best available grade (Spectrograde); 1,2-dimethoxyethane, HPLC grade, was from Fluka A.G.

Cells. Adherent mouse splenocytes were obtained from 30–50 g Swiss albino strain mice. Spleens were removed aseptically and gently teased through a 80-μm stainless-steel grid partially immersed in heparinized (5 U/ml) Hanks' medium at 20°C. After cell collection and erythrocyte lysis (8), the splenocytes were resuspended in Medium 199 containing 20% (v/v) heat-inactivated fetal calf serum supplemented with glutamine (685 μM), heparin (5 U/ml), and $NaHCO_3$ (8.9 mM). The cell suspension (5 to 6 × 10^6 cells/ml) was

[2] Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

0003-2697/86 $3.00

transferred to glass petri dishes and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 (final pH 7.2). Nonadherent and loosely attached cells were then removed by rinsing the dishes with fresh medium.

Mice resident peritoneal cells were obtained by peritoneal lavage with 5 ml of heparinized (5 U/ml) phosphate-buffered saline (PBS), pH 7.4. The cells were centrifuged at 200*g* (15 min) and resuspended (10^6 cells/ml) in DMEM supplemented with heat-inactivated newborn calf serum (20% v/v) and heparin (5 U/ml). Cells thus obtained were used for phagocytosis assays either as suspensions or were incubated and allowed to adhere (2 h) to plastic petri dishes. Viability of the cells estimated by trypan blue exclusion was routinely superior to 95%.

Phagocytosis assays. Polystyrene latex beads (1.1-μm diameter) were resuspended in fetal calf serum by a brief treatment in a sonicator bath and left 24 h at 4°C. The opsonized beads (400 particles/cell) were added to peritoneal macrophages either adherent (1.75×10^6 cells/well of 2 cm^2) or in suspensions (0.6×10^6 cells/ml in 25-ml flasks). After the incubation time the cells were washed four times with cold PBS, in order to remove nonadherent particles, and collected in PBS containing 0.1% (w/v) emulphogene BC 720. The lysate was centrifuged for 10 min at 10,000*g* and the pelleted particles were washed twice the same way and finally resuspended in water for latex analysis.

Phagosome isolation. Adherent splenocytes and opsonized beads (2 to 3×10^3 particles/cell) were incubated at 37°C for 60 min. After phagocytosis the excess beads were removed by vigorous washing (four times) with cold (4°C) PBS (pH 7.4) and the cells were scraped with a rubber policeman into the same medium. After homogenization in a Dounce homogenizer the suspension was adjusted to 30% (w/v) sucrose and the phagosomes were isolated by floatation essentially as described in (6).

Polystyrene latex quantification by fluorescence. Polystyrene latex particles associated with cells or phagosomes (100 μl of an aqueous sample containing between 0.5 and 20 μg latex) were added to 1.9 ml of 1,2-dimethoxyethane (redistilled) and the mixture was vortexed. When needed, residues were removed by a short centrifugation (5 min) at 10,000*g*. The fluorescence was then determined in thermostated (25°C) cells of 1-cm path length with a Jobin Yvon (model JY-3D) fluorometer equipped with a xenon lamp. The slitwidth on excitation and emission monochromators was routinely set at 4 nm; excitation and emission wavelengths were set at 268 and 335 nm, respectively. A standard curve was established under the same conditions from the purchased stock according to the manufacturer's indications (1 μg of latex corresponded to 1.76×10^6 particles).

RESULTS

Absorbance and fluorescence properties of polystyrene latex. When dissolved in an organic solvent such as dioxane, polystyrene presents an absorbance maximum at 259 nm. At a concentration of 1 μg/ml the absorbance is about 2.3×10^{-3} (1). Polystyrene solutions are also fluorescent. In hexane the emission maximum is at 330 nm (λ_{ex} = 265 nm) and the quantum yield is equal to 0.03 (9). We have measured the fluorescence of the latex beads in several solvents miscible with water which present a good solubilization of polystyrene (dioxane, 1,2-dimethoxyethane, diethylene glycol, and tetrahydrofuran). Due to its very low fluorescent background we have selected 1,2-dimethoxyethane as the solvent for our studies. Several other solvents contain antioxidants which contribute to the fluorescence at the wavelengths of interest. In 1,2-dimethoxyethane the maximal wavelengths of excitation and emission were, respectively, 268 and 335 nm.

Quantification of the latex beads by fluorescence. The polystyrene latex particles were solubilized in 1,2-dimethoxyethane/H_2O (95:5, v/v) and the fluorescence was measured in a range of 0.25 to 50 μg/assay. Figure 1 rep-

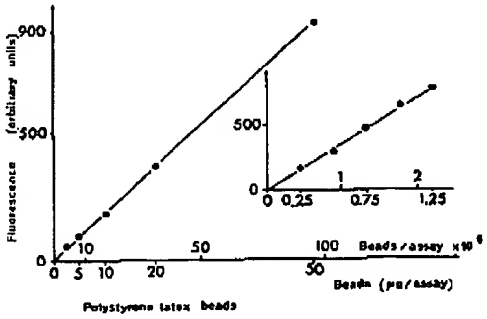

FIG. 1. Quantification of polystyrene latex beads by fluorescence. Latex beads (1.1 μm) were solubilized in 1,2-dimethoxyethane–water (95:5, v/v) and the fluorescence was determined at 335 nm (λ_{ex} = 268 nm) in thermostated cells at 25°C.

resents the relationship between the fluorescence at 335 nm and the concentration of latex beads. The assay, which can be easily miniaturized, proved highly sensitive and the fluorescence intensity is linear with the number of beads (r = 0.999 in the 5–50 μg range and r = 0.998 in the 0.25–1.25 μg range). No inner filter effect was noticeable up to 50 μg polystyrene latex/ml; departure from linearity became detectable only above 100 μg/ml (not shown). The present method compares favorably with the spectrophotometric assay of polystyrene latex beads, which was accurate in the 20–200 μg/assay range (1,7).

Application to phagocytosis quantification and to phagosome isolation. The high sensitivity of the fluorescent quantification of polystyrene allowed the determination of phagocytosis by a relatively small number of cells (less than 2×10^5). Opsonized latex beads were given to suspensions of mice peritoneal macrophages, and the extent of phagocytosis as a function of time was determined at 37°C. After the incubation period the cells were washed with phosphate-buffered saline by successive centrifugations. Latex determination in the supernatants indicated that four washes were needed to eliminate free or loosely attached particles. To distinguish strongly adsorbed beads from ingested ones, parallel experiments were performed at 4°C, a temperature known to block phagocytosis. The uptake of the latex beads by the cells as determined by the fluorometric method is shown in Fig. 2. To avoid the contribution of the intrinsic fluorescence of the cells to latex measurements, the particles were obtained from cells by digestion in emulphogene BC 720 (a nonionic detergent) and washed twice by centrifugation in PBS. Under our experimental conditions phagocytosis reached a maximum after 60 min at 37°C with an average uptake of 20 beads/cell. Similar results were obtained with adherent peritoneal macrophages (not shown).

Since phagocytosis is known to be stimulated by mediators such as lymphokines or tuftsin (10), we have tested the sensitivity of our phagocytosis assay in such cases. Adherent peritoneal macrophages were incubated at 37°C with increasing amounts of tuftsin. An important increase in phagocytized beads was observed when phagocytosis was measured at 60 min (Fig. 3). A maximal stimulation was reached with 100 ng tuftsin/ml with a fourfold increase in latex bead uptake.

An interesting aspect of latex bead phagocytosis is the possibility to isolate phagosomes and study their composition, e.g., ectoenzyme

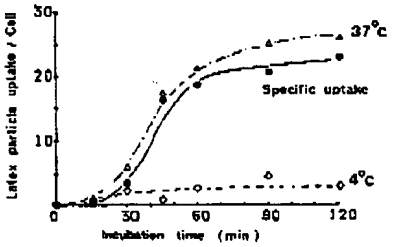

FIG. 2. Phagocytosis of latex beads by mouse peritoneal macrophages. Cells (25 ml at 0.6×10^6 cells/ml) in Dulbecco's modified Eagle's medium–newborn calf serum (20% v/v) were incubated with latex beads (400 particles/cell) for varying times at 37°C (△) or 4°C (◇). Cell aliquots were washed four times with phosphate-buffered saline, pH 7.4, and resuspended in 2 ml of 1,2-dimethoxyethane–H_2O (95:5, v/v); the fluorescence was determined against a standard curve. Specific uptake (●) represents the difference between the beads associated with cells at 37 and 4°C.

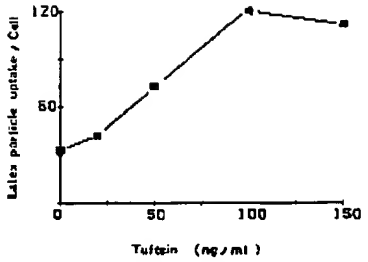

FIG. 3. Stimulation of phagocytosis by tuftsin. Adherent peritoneal macrophages (4.8×10^6 cells/9.6 cm^2 well) in Dulbecco's modified Eagle's medium–newborn calf serum (20% v/v) were incubated at 37°C for 60 min with latex beads (350 particles/cell) and increasing amounts of tuftsin. After four washes with cold phosphate-buffered saline, the cells were collected and the fluorescence of the latex was determined against a standard curve. Values given are means of two determinations.

activities, associated with the phagosomal membrane (6). Our latex determination by fluorescence proved particularly useful in such experiments for the quantification of limited amounts of phagosomal fractions. Adherent splenic macrophages were incubated with latex beads and the phagosomes were isolated, as described under Materials and Methods, by a floatation technique using a discontinuous sucrose density gradient. The phagosome fraction was assayed for its latex and enzyme content, e.g., internalized nucleotide pyrophosphatase, an ectoenzyme (11) (Table 1). Polystyrene latex was easily quantified by our method and contrary to the spectrophotometric assay (6) no interference by sucrose was observed; i.e., up to 30% sucrose in the aqueous phase of the present assay medium did not significantly affect (less than 2%) the fluorescence of the latex beads. The contribution to the fluorescence by the proteins adsorbed on the beads was minimal; in control experiments the phagosomes were digested in 0.1 M NaOH for 16 h at 25°C and washed and this treatment led to less than 5% decrease in fluorescence compared to untreated phagosomes.

DISCUSSION

Our aim in developing a sensitive fluorescent assay for the determination of polystyrene latex beads was twofold: (i) rapid quantification of latex particle phagocytosis on small cell samples and (ii) quantification of the phagosomal fractions allowing the study of enzyme activities associated with these vesicles. The use of the intrinsic fluorescence of polystyrene in organic solvents such as 1,2-dimethoxyethane proved to be a valuable method for the quantification of latex beads. Its sensitivity in

TABLE 1

RECOVERY OF LATEX AND NUCLEOTIDE PYROPHOSPHATASE IN PHAGOLYSOSOMES FROM SPLENIC MACROPHAGES

Fraction	Latex (μg)	Proteins (μg)	Nucleotide pyrophosphatase (nmol/min)
Homogenate	39.5 ± 13.4 (100)	151 ± 31 (100)	0.427 ± 0.263 (100)
30%	ND	128 ± 27 (74)	0.282 ± 0.150 (76)
10/20% IF	27.3 ± 6.1 (72)	17 ± 4 (19)	0.035 ± 0.009 (10.5)
Recoveries (%)	72 ± 14	93 ± 14	86 ± 20

Note. Adherent splenic macrophages (about 3×10^6 cells in a petri dish of ϕ 20 cm) were exposed to 2 to 3×10^3 latex beads/cell for 60 min at 37°C. Phagolysosomes were isolated as described under Materials and Methods. Fractions analyzed consisted of the cell homogenate after phagocytosis, the 30% sucrose layer of the discontinuous gradient after centrifugation, and the phagolysosomal fraction at the 10/20 % sucrose interface. Latex was quantified with the fluorometric technique using about 0.2–0.8 μg/assay. The nucleotide pyrophosphatase activity was assayed fluorometrically with pyridine-1,N^6-ethenoadenine dinucleotide as substrate (12). The values are the means (±SD) of four experiments. The number in parentheses correspond to the respective percentages. ND, not detectable.

the microgram range (i.e., 10^6 beads of 1.1-μm diameter) is at least one order of magnitude greater than that of the conventional spectrophotometric method (1,7) and circumvents the need for large cell quantities. Internalized beads were distinguished from those merely adsorbed to the cell surface by phagocytosis inhibition at 4°C. Using our method, phagocytosis was routinely determined with about 2×10^5 macrophages.

In other studies we have followed the fate of several enzymes, e.g., NAD^+ glycohydrolase and nucleotide pyrophosphatase, associated with the outer surface of macrophages during latex bead phagocytosis. To evaluate the degree of internalization of these ectoenzymes we have isolated the phagolysosomes (Table 1; C. D. Muller, to be published). The present determination of latex allowed an easy quantification of the phagosomal fraction in sample sizes suitable for the use of sensitive fluorometric assays of the enzymes associated with these fractions, e.g., nucleotide pyrophosphatase (12), NAD^+ glycohydrolase (13), esterases, and other hydrolases (8,14). In this particular experiment one can estimate that an average of 24 beads were phagocytized per macrophage, a value compatible with the observed extent of internalization of the ectonucleotide pyrophosphatase; i.e., assuming that each bead is ingested and closely surrounded by plasma membrane, this would require internalization of an average of 100 μm^2 of membrane and, according to (15), approximately 10–12% of total cell surface. Recently the use of fluorescent latex beads has been described for the study of phagocytosis (16,17). These methods, which present a comparable sensitivity, will certainly be convenient for direct phagocytosis quantification, but because of spectral overlaps might interfere with the fluorometric assays of phagolysosomal associated enzyme activities.

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